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RESEARCH ARTICLE

Phylogeny does not predict the outcome of heterospecific pollen–pistil interactions in a species-rich alpine plant community

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Abstract

Premise: Co-occurring plant species that share generalist pollinators often exchange pollen. This heterospecific pollen transfer (HPT) impacts male and female reproductive success through pollen loss and reductions in seed set, respectively. The resulting fitness cost of HPT imposes selection on reproductive traits (e.g., floral color and shape), yet we currently lack strong predictors for the post-pollination fate of heterospecific pollen, especially within community and phylogenetic contexts.

Methods: We investigated the fate of heterospecific pollen at three distinct stages of plant reproduction: (1) pollen germination on the stigma, (2) pollen tube growth in the style, and (3) fertilization of ovules. We experimentally crossed 11 naturally co-flowering species in the subalpine meadows of the Colorado Rocky Mountains, across a spectrum of phylogenetic relatedness. Using generalized linear mixed models and generalized linear models, we evaluated the effect of parental species identity and phylogenetic relatedness on pollen tube growth at each reproductive stage.

Results: We found that heterospecific pollen tubes can germinate and grow within pistils at each reproductive stage, even when parental species are >100 My divergent. There was no significant effect of phylogenetic distance on heterospecific pollen success, and no evidence for a mechanism that suspends heterospecific pollen germination or pollen tube growth within heterospecific stigmas or styles.

Conclusions: Our results show that even in communities where HPT is common, pre-zygotic post-pollination mechanisms do not provide strong barriers to interspecific fertilization. HPT can result in the loss of ovules even between highly diverged plant species.

KEYWORDS

alpine plants, heterospecific pollen transfer, *Pedicularis*, pollen-pistil interactions, pollen tubes, phylogenetic distance

Many plant species use animal pollinators as vectors to efficiently disperse pollen between individuals. In species-rich plant communities, the use of shared pollinators by different plant species can result in heterospecific pollen transfer (HPT), a process that generally reduces both male and female reproductive success (Ashman and Arceo-Gómez, 2013). Much of the incredible reproductive diversity of flowering plants, such as differences in floral phenology, scent, shape, size, color, and rewards, increases pollinator fidelity and reduces HPT (Harder and Johnson, 2009; Armbruster et al., 2014).

The negative fitness consequences of HPT can occur at various stages of the reproductive cycle. For example, in the pistil (i.e., pollen recipient) reproductive tract, heterospecific pollen or pollen tubes can interfere with conspecific pollen on the stigmatic surface or within the style to reduce the probability of conspecific fertilization, even if heterospecific pollen tubes are unable to reach and fertilize ovules (i.e., exhibits post-pollination prezygotic isolation). Decreases in seed set following HPT are primarily viewed as a consequence of heterospecific pollen grains or tubes physically blocking conspecific pollen or tubes at the stigmatic surface

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or within the style, respectively (Kohn and Waser, 1985; Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019). However, when heterospecific pollen tubes can reach and fertilize ovules, a further cost to the maternal plant can be incurred from the loss of ovules to unfit or aborted embryos (i.e., post-pollination postzygotic isolation). In either case, the maternal plant may suffer a fitness cost from HPT, but the extent of this loss is expected to be greater when heterospecific pollen tubes have traversed farther into the female reproductive tract. Tracking the fate of heterospecific pollen transferred between species in plant communities can thus be informative about the fitness costs of interspecific reproductive interactions and, in turn, how these costs shape plant communities.

Despite the assumed importance of HPT in driving reproductive divergence among flowering plants, many questions remain about its extent and impact. Does pollen from closely related species cause more detrimental effects than pollen from more distant relatives? At what stage of pollination (e.g., pollen deposition, pollen tube growth, fertilization) is heterospecific pollen growth typically arrested? While these questions have been investigated in crossing studies among a few economically important groups of plants, we generally lack knowledge of the fate and impact of HPT among diverse plant species in natural communities. Direct measurements of the frequency of HPT and its costs can be challenging, even in controlled experiments. A number of approaches have been employed to measure the effects of HPT during reproduction, including quantifying (1) pollen deposition on foreign stigmas, (2) pollen germination on foreign stigmas, and (3) the impact of heterospecific pollen tubes on seed set in pure and mixed pollen loads. Studies have consistently shown that HPT is frequent in natural communities (Feinsinger et al., 1986; Bartomeus et al., 2008; Montgomery and Rathcke, 2012; Tong and Huang, 2016); however, measurements that examine the effects of HPT have been less conclusive.

The impact of HPT on female reproductive success has been measured from experiments that examine developing embryos or seed set. In these experiments, pollen is applied manually to compare the results of different treatments, often using conspecific versus mixed or layered pollen loads (i.e., heterospecific pollen followed by conspecific pollen). While this type of experiment has often shown negative effects of HPT on seed set (Kanchan and Chandra, 1980; Jakobsson et al., 2008; Huang et al., 2016), it has also sometimes shown no effect (Waser, 1978; Armbruster and McGuire, 1991; Kwak and Jennersten, 1986, 1991; Caruso, 2000; Tscheulin et al., 2009). These studies focused primarily on the outcome of heterospecific pollination interactions rather than the mechanism by which seed set is reduced, often assuming that the style is physically clogged without any direct observation of pollen tubes in the style. In this context, the historical focus on physical blocking mechanisms may overshadow alternative processes resulting from HPT, such as the usurpation of ovules by heterospecific pollen.

Where reproductive barriers are operating along the reproductive tract will be directly related to the mechanism by which HPT reduces seed set. Hogenboom (1975) theorized that unsuccessful heterospecific pollination interactions will be caused either by (1) self-incompatibility mechanisms (such as *S* alleles) or (2) from genetic incongruities (e.g., epistasis) between pollen and pistil, which arise over time as mutations accumulate that cause pollen–pistil recognition systems to diverge. Self-incompatibility mechanisms may contribute to interspecific isolation under the "SIxSC rule", where self-incompatible species reject pollen from self-compatible relatives on the stigma, but not vice-versa (Hirose et al., 1995; Goodwillie and Ness, 2013; Baek et al., 2015). Arrest of heterospecific pollen tubes by *S* alleles will occur at the stigmatic surface (as in sporophytic self-incompatibility; Nasrallah and Nasrallah, 1993) or in the stigma or style (as in gametophytic self-incompatibility; Luu et al., 2000) thus could result in mechanical clogging of the style. If non-functioning of the pollen–pistil relationship is the result of genetic incongruities, however, then reproductive barriers may operate anywhere in the reproductive tract. Where HPT is common, late-acting (e.g., post-zygotic) reproductive barriers are the most costly, as ovule usurpation results in the greatest loss of resources. Selection is therefore expected to favor earlier-acting reproductive barriers, such as the recognition of heterospecific pollen at the stigmatic surface or rejection or suspension of heterospecific pollen tube growth in the style. These barriers should be the strongest when a cross is less likely to be viable, which is the case when phylogenetically distant relatives exchange pollen.

Whether or not heterospecific pollen transferred between species is capable of usurping ovules is a fundamental question that can inform our understanding of the evolutionary and ecological importance of HPT. This question has most often been investigated at the level of intrageneric crosses, which show that hybrid seeds are often formed but commonly inviable (Coughlan, 2023), demonstrating that ovule usurpation is frequently possible, at least among close relatives. However, crossing studies among more distantly related species (e.g., greater than tens of millions of years diverged) are much less common. Consequently, we have little evidence from which to assess the hypothesis that phylogenetic distance can predict the impact of HPT. Further empirical studies examining the fate of heterospecific pollen tube growth within a community context, especially in systems where HPT is common among both close and distant relatives would give insight into the true impact of HPT.

Alpine meadows represent an interesting system in which to investigate heterospecific pollination because they often harbor high plant species diversity (Myers et al., 2000) and the growing seasons are short, such that many plant species flower synchronously and utilize shared pollinators (Hughes and Atchison, 2015). Alpine lineages are often characterized by high floral morphological diversity, which may evolve as a consequence of character displacement to reduce reproductive interference in an environment where the opportunity for phenological

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divergence is limited (Körner, 1995; Eaton et al., 2012; Hughes and Atchison, 2015). Here, we performed a series of crossing experiments to examine and quantify heterospecific pollen tube growth across a spectrum of phylogenetic distances among sympatric plant species in an alpine plant community. We asked whether phylogenetic distance between pollen and pistil is a significant predictor of heterospecific pollen fates at three distinct stages of reproduction (Figure 1). We asked: (1) Can heterospecific pollen germinate on stigmas? (2) How far into the reproductive tract can heterospecific pollen tubes grow? (3) Can heterospecific pollen tubes lead to development of embryos following fertilization? We sought to understand if and when barriers to reproduction act on heterospecific pollen

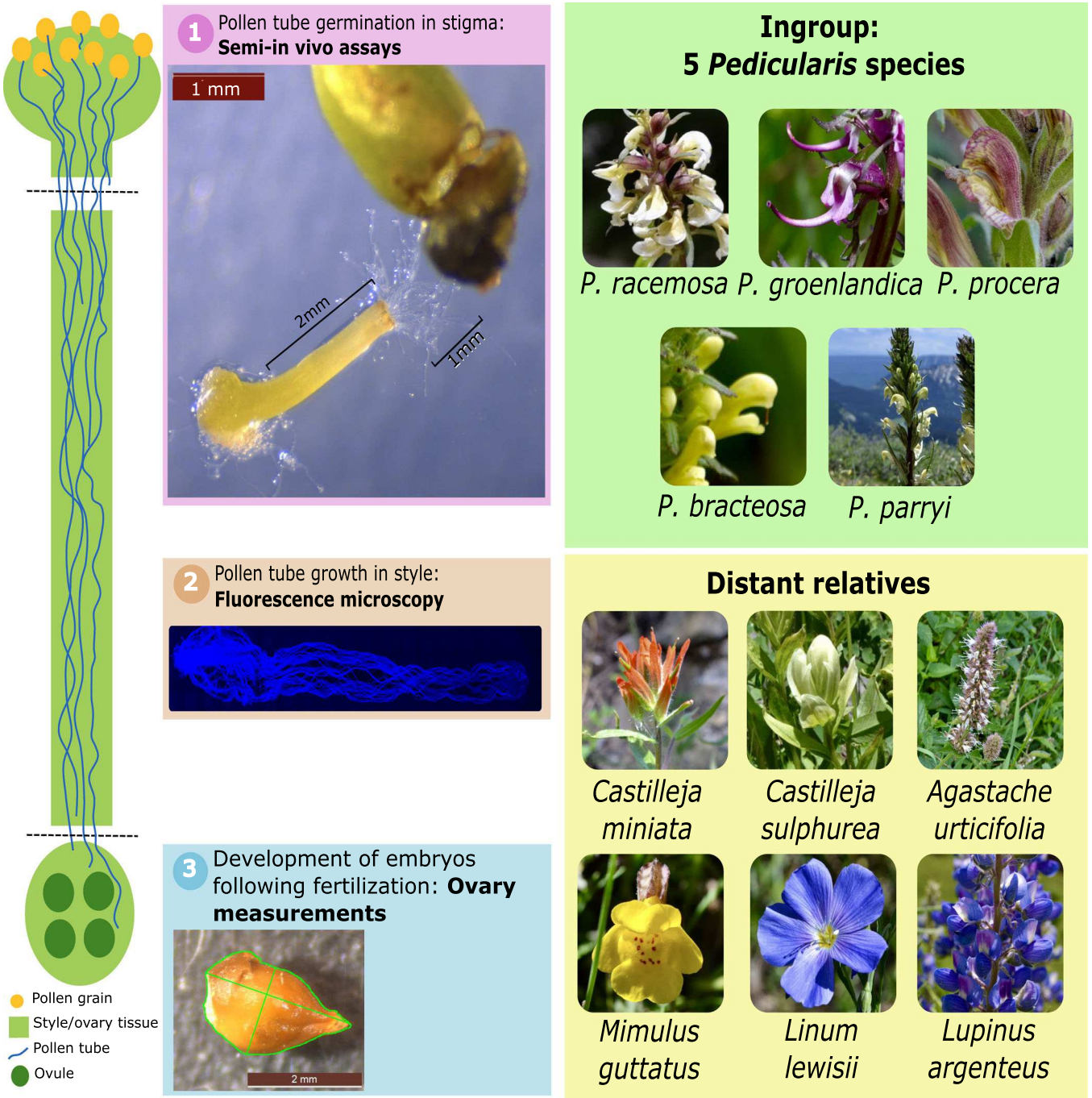


FIGURE 1 Examining the fate of heterospecific pollen during three distinct stages of plant reproduction. We evaluated (1) pollen germination in semi-in vivo pollen tube assays (described in Materials and Methods), (2) heterospecific pollen tube growth using fluorescence microscopy, and (3) fertilization using ovary size measurements. We quantified how heterospecific pollen fate varied with respect to the genetic relatedness between the parents (pollen species and pistil species). Our phylogenetic ingroup consisted of five species of North American *Pedicularis* (green box), which we crossed to within-family, within-order, and very-distant relatives (yellow box).

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tubes, hypothesizing that barriers that facilitate the rejection of foreign pollen will act earlier in crosses involving more distantly related species pairs.

MATERIALS AND METHODS

Study system

We focused on several sympatric North American plant species in the genus *Pedicularis* (Orobanchaceae) and their alpine plant community. We chose *Pedicularis* as a focal clade because reproductive interference via HPT has been hypothesized to drive selection on floral morphological divergence in the genus (Li, 1951; Ree, 2005; Eaton et al., 2012). *Pedicularis* species exhibit striking differences in flower color, shape, and size. Most notably, variations in the galea and style affect pollen placement and pollen–pistil interactions, respectively. The galea is a hood or beak-like structure formed by a fusion of the two upper petals, which surrounds the anthers and directs pollen placement onto a specific part of a visiting bee's body (Grant, 1994; Huang et al., 2016; Tong and Huang, 2016). Differences in the length and curvature of the galea among *Pedicularis* species play an important role in reducing HPT, especially in species-rich communities (Eaton et al., 2012). In addition to having this pre-pollination barrier, *Pedicularis* also exhibits significant variation in style lengths, which range over an order of magnitude in size and have been shown to act as a post-pollination prezygotic reproductive barrier (Tong and Huang, 2016). Despite these floral differences, observational and experimental pollination studies in both North America and China have demonstrated frequent pollinator switching and HPT among co-occurring *Pedicularis* species (Macior, 1970, 1983, 1995; Fang and Huang, 2013; Huang et al., 2016; Tong and Huang, 2016). *Pedicularis* is thus a well-established system in which to examine the evolutionary consequences of HPT.

All experiments were performed at the Rocky Mountain Biological Laboratory in Gothic, Colorado, United States during June–July 2022 and June–August 2023. We used a total of 11 species for crossing experiments (Figure 1), with the focal "ingroup" consisting of the five commonly occurring *Pedicularis* species in the area: *P. groenlandica*, *P. bracteosa*, *P. procera*, *P. racemosa*, and *P. parryi*. The other six species were selected to span a range of close to distant relationships to *Pedicularis*, all of which occur abundantly and flower synchronously: *Castilleja miniata*, *C. sulphurea* (Orobanchaceae), *Agastache urticifolia* (Lamiaceae), *Mimulus guttatus* (Phrymaceae), *Linum lewisii* (Linaceae), and *Lupinus argenteus* (Fabaceae) (herbarium voucher information can be found in Appendix 1). All species occur in alpine and subalpine meadows (2700–3700 m a.s.l.), are at least partially bumblebee pollinated and share pollinators. Additionally, all species included are self-compatible with the exception of those species in *Castilleja*, which have been referred to as "partially self-compatible" (Hersch and Roy, 2007).

Semi-in vivo pollen tube growth experiment

To evaluate the germination of heterospecific pollen on a stigma, we used semi-in vivo pollen tube growth assays (Qin et al., 2009). We performed an 11 × 11 series of reciprocal crosses between all study species, including a treatment representing a same-species (conspecific) outcross. To ensure virgin stigmas, we collected unopened flower buds that were close to opening. "Bud pollination" has been used in agricultural settings to increase hybridization success: early-stage buds have nonreceptive stigmas that have yet to develop species recognition mechanisms (Nasrallah and Nasrallah, 1993; Bhat and Sarla, 2004). We aimed to capture potential species recognition mechanisms in action, so we collected late-stage buds with receptive stigmas and anthers close to maturity. Flower buds were collected, dissected, and plated the same day to ensure flower freshness. We tested stigma receptivity using hydrogen peroxide (Kearns and Inouye, 1993) applied to five buds from each batch of buds collected. If all five stigmas were determined to be receptive, then buds of that size were used for experimentation. We carefully removed the anthers to use as pollen treatments in the experiment, then removed the ovary to be used as an attractant and cut the style such that it was no more than 2 mm long. We dipped the stigma in fresh pollen (collected within 24 h of pollination, stored at 4°C), making sure it was completely covered, and placed it on pollen tube growth medium in a petri dish with the truncated style end within 1 mm of its own ovary (Figure 1, stage 1). Each stigma received pollen from only one species (i.e., pure pollen loads).

The pollen tube growth medium consisted of 0.01% w/v boric acid, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10% w/v sucrose, and 1.5% w/v agarose, and had a pH of 7.5. We confirmed that the suitability of the pollen tube growth medium did not vary significantly among our study species by first applying pollen from all species to individual dishes and observing pollen germination in vitro. All species had >80% germination success in this test, and so we used the same growth medium for all species in our study. We incubated the petri dishes in a growth chamber, with 18 h of daylight at 23°C and 6 h of darkness at 18°C for 48 h, then used a dissecting microscope (maximum 40× magnification) to score each assay for the presence of pollen tubes growing out of the truncated end of the style.

Due to differences in pollen grain and stigma size, it was infeasible to quantify the number of pollen grains deposited on each stigma; thus, the number of pollen tubes growing out of the truncated style could not reliably be compared across treatments. We therefore chose to record pollen tube growth as a binary outcome: pollen tubes present or absent. These assays were performed in both 2022 and 2023, and data from both years were pooled for analysis. We had at least 10 total replicates of each cross type across both years and calculated the proportion of replicates in which pollen tubes grew out of the excised style for each cross-type.

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In vivo pollen tube growth experiment

We conducted field crosses using a six-species subset of our study species: *P. groenlandica*, *P. procera*, *P. bracteosa*, *Castilleja miniata*, *Mimulus*, and *Lupinus*. To ensure virgin stigmas for all six species, we placed pollination bags over unopened buds early in the flowering season. Once flowers opened (approximately 3 weeks following initial bagging, varied by species), we manually pollinated flowers using a paintbrush. All pollen used in this experiment was collected no more than 24 h before pollination and stored at 4°C until use.

Individual plants received pollen from a single species to decrease the chance of contamination, and multiple individuals were included in each pollen treatment. We pollinated plants that were spread across the population (i.e., we did not use one pollen treatment all in one area). In *Mimulus* and *Lupinus*, stamens and pistils are of similar length, so autonomous self-pollination was prevented by carefully emasculating flowers before anthesis. We additionally included a control "unpollinated" treatment, and these flowers were also emasculated. All open flowers on a single inflorescence were pollinated, and unopened, unpollinated flowers were removed from the inflorescence. Due to differences in inflorescence size, the number of flowers that were hand-pollinated varied across species. We pollinated between 14 (unpollinated *Mimulus*) and 86 (*P. groenlandica* × *P. groenlandica*) flowers per pollination treatment, and pollination bags were replaced immediately following pollination. We collected the flowers 72 h post-pollination and stored them immediately in ethanol. Before imaging, we carefully removed the pistil from each collected flower and cut the ovary off. Ovaries and styles were then stored separately in ethanol until imaging (see section Ovary response experiment for details on ovary imaging).

To visualize pollen tube length in the style after field pollination, we used 0.1% w/v aniline blue in 0.1 M K_3PO_4, pH 6.5) to visualize fluorescence from callose in the walls of pollen tubes (Martin, 1959). First, we rinsed the pistils three times with distilled water to remove excess ethanol, then we incubated the pistils in 1 M NaOH to clear and soften pistils for imaging. Because each species varied in pistil thickness and waxiness, each pistil type required a different incubation period: *Pedicularis* and *Castilleja* were incubated for 30 min, *Mimulus* for 20 min, and *Lupinus* for 40 min. Pistils were then rinsed three times with distilled water, then placed in aniline blue overnight at 4°C. To avoid batch effects, we did not stain all pistils from a single species on the same day or with a single batch of stain. Each stained pistil was additionally labeled with a code before imaging so the observer was blind to the cross type.

Pistils were individually mounted on microscope slides and imaged at 20× magnification using a Zeiss confocal LSM700 epiflourescence microscope with 350 nm excitation and 450 nm emission. We scored each pistil according to the location of the pollen tubes in the pistil: (1) Pollen grains on stigma did not germinate, and no pollen tubes were observed; (2) pollen tubes were in the stigma or top of the style; (3) pollen tubes reached >50% of the distance down the style; and (4) pollen tubes at the base of the style. For each species, we also measured style length and stigma depth (vertical distance from the stigma top to top of the style), which have been shown to be associated with differences in pollen tube growth success in heterospecific crosses (Wang et al., 2016). To get mean values for style length and stigma depth for the six species included in this experiment, we randomly selected 20 images associated with each species and measured style length and stigma depth using ImageJ2 software (Rueden et al., 2017).

Ovary response experiment

We measured the size of ovaries as a proxy for detecting ovule fertilization in a pollination treatment. Ovaries were photographed on a dissecting microscope with a Leica camera at a maximum of 40× magnification. All images included a scale bar for accurately measuring ovary length, width, and area using ImageJ2. Because ovaries are not always evenly shaped, we measured the length of the ovary as the longest dimension of the ovary and the width at the widest section perpendicular to the measured length. Area was approximated by tracing the perimeter of the ovary. Lengths and widths are reported in Appendix S1: Figure S1. Each of the three dimensions were measured individually for each ovary image, and ovaries from each species were measured by the same researcher to control for differences in measurement strategy.

Statistical analyses

To evaluate the relationship between relative phylogenetic distance and our three measurements of pollen fate (germination, pollen tube growth, and fertilization), we modeled pollen tube length (semi-in vivo and in vivo pollen tube growth experiments) and ovary area (as a proxy for fertilization) as a function of relative phylogenetic distance in each of the three experiments. To calculate relative phylogenetic distance, we first generated a phylogeny from ITS sequences downloaded from NCBI (Appendix S2: Table S1), aligned the sequences with the msa function in the R package msa (Bodenhofer et al., 2015), then calculated a distance alignment using the function dist.alignment on the multispecies ITS sequence alignment in the R package seqnir (Charif and Lobry, 2007). We inferred a tree topology using neighbor joining via the BIONJ algorithm (Gascuel, 1997) using the function bionj in the R package ape (Paradis and Schliep, 2019). We then transformed the tree to be ultrametric with time-calibrated nodes using divergence time estimates from timetree.org (Kumar et al., 2022, Appendix S2: Table S2) as calibrations for the penalized likelihood approach implemented in the chronos function in ape. With the resultant tree, we calculated the patristic phylogenetic distance between each pair of species with the distTips function in the adephylo package (Jombart et al., 2010) and divided each distance value by the maximum patristic distance to obtain a relative phylogenetic distance measurement.

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For the semi-in vivo experiment, we used generalized linear mixed models (GLMMs) to fit a logistic regression. Because different species responded to the assay conditions differently, we modeled the proportion of trials in which pollen tubes germinated in the stigma as a function of both genetic distance and pollen germination success during conspecific crosses. We additionally tested for species-level effects by evaluating the significance of including pistil and pollen identity as random effects in the models. Models were fit using the lme4 package in R (Bates et al., 2015).

Because pollen tube growth in the style was recorded as a categorical outcome, we fit an ordinal regression using the ordinal package in R (Christensen, 2023) to evaluate the relationship between phylogenetic distance and pollen tube length in the style. The ordinal regression models the latent states of pollen tube length along the pollen tube pathway (i.e., 23% of the way, 87% of the way) that were not directly recorded. We modeled pollen tube length down the pistil as a function of relative phylogenetic distance and pollen tube growth in conspecific crosses, evaluating the significance of pistil and pollen identity when included as random effects. We separately evaluated the correlation between pollen tube length in the style and difference in average style lengths between the pollen species and pistil species using Gaussian distributed linear regression models.

To evaluate changes in ovary size following the different pollination treatments, we fit a Gaussian distributed generalized linear model. Because each species had differently sized ovaries before any manipulation, we normalized ovary area by dividing each measurement by the average ovary area for each pistil species in the unpollinated treatment and modeled the normalized area as a function of genetic distance and area following conspecific pollination. We additionally evaluated the significance of pistil and pollen identity as random effects. We compared variance in ovary size following pollination treatments with variance during the unpollinated treatment using a Fisher's F test. We also evaluated the correlation between pollen tube progression in the pistil and ovary size change using a Gaussian distributed linear regression model.

To select the best-fitting model in each experiment, we compared models using Akaike's information criterion (AIC), taking the model with the lowest AIC score as the best-fitting model. Models that differed by <2.0 AIC points from the lowest score were determined not to be quantitatively different from one another. In such a case, the most parsimonious (i.e., model with the fewest terms) was chosen as the best-fit model.

RESULTS

Semi-in vivo pollen tube assay results

Phylogenetic distance between pollen and pistil species was not a strong predictor of pollen tube germination and growth through the stigma. The best-fitting model shows a weakly negative but nonsignificant relationship (slope = −1.169, $P = 0.183$; Table 1; Figure 2A, B). All conspecific crosses

TABLE 1 Model comparisons of the generalized linear mixed models for all three experiments. We compared models using AIC values. We report the slope associated with relative phylogenetic distance and its P-value for each model. We examined the effect of Genetic Distance (GD) and conspecific pollen germination, pollen tube growth, or ovary size change, respectively (CON) on heterospecific pollen fates. We additionally included random effect (RE) terms to evaluate the role of pollen or pistil identity in heterospecific pollen fates.

Experiment	Model	AIC	Slope	P
Semi-in vivo pollen tube experiment (binomial GLMM)	**GD + CON + RE(pollen)**	**530.19**	**−1.169**	**0.183**
	GD + CON + RE(pollen) + RE(pistil)	533.66	−1.353	0.145
	GD + CON	559.7	−0.936	0.006
	GD + CON + RE(pistil)	564.37	−0.912	0.019
	Null	625.25		
Fluorescence microscopy experiment (ordinal regression)	**GD + CON**	**2403.8**	**−0.091**	**0.78**
	GD + CON + RE(pollen) + RE(pistil)	2402.2	−0.01	0.867
	GD + CON + RE(pistil)	2402.5	−0.394	0.343
	GD + CON + RE(pollen)	2403.6	0.25	0.551
	Null	2424.1		
Ovary measurements experiment (Gaussian GLM)	**GD + CON + RE(pistil)**	**1701.168**	**−0.758**	**0.129**
	GD + CON + RE(pollen) + RE(pistil)	1704.7	−0.585	0.099
	GD + CON	1787.536	−0.492	0.002
	Null	1792.35		
	GD + CON + RE(pollen)	1810.294	−0.042	0.019

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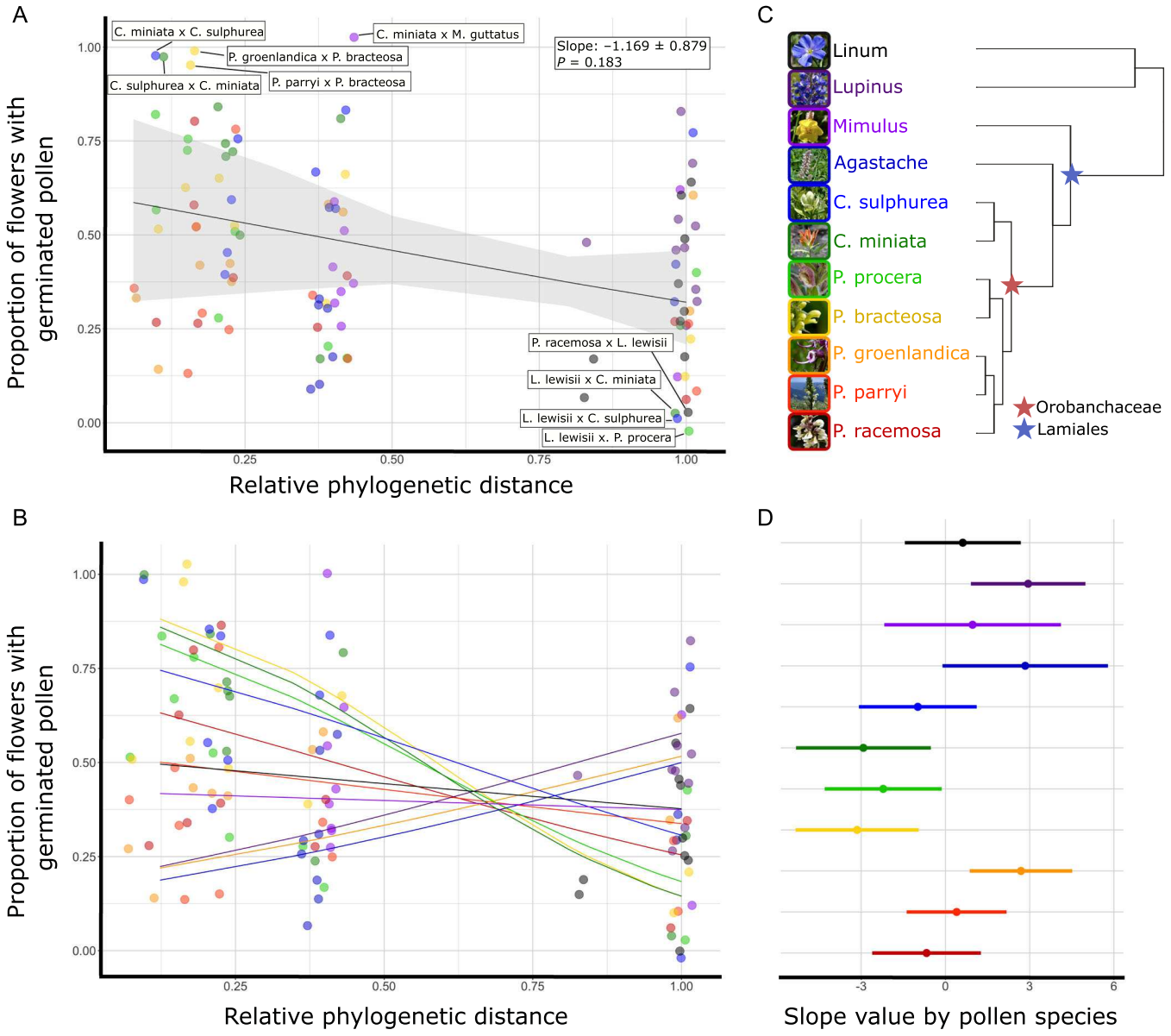


FIGURE 2 Results from semi-in vivo pollen tube experiment. Colors correspond to pollen parent identity. (A, B) Each point represents the proportion of replicate trials in which we observed pollen tube germination for heterospecific crosses. (A) Best-fit model for pollen tube germination (slope = −1.665, $P = 0.156$, see Table 1), shaded error bars are 95% confidence intervals. Crosses that exhibited exceptionally low (<0.1) and high (>0.9) proportion germination are individually labeled. (B) We show significant variation in the slope values (i.e., relationship between relative phylogenetic distance and pollen tube growth) according to pollen species identity. (C) Phylogeny of the 11 study species included in the semi-in vivo pollen tube germination experiment. (D) Dot plot of estimations of slopes by pollen species. The point represents the point estimate of the slope; the lines show the conditional standard deviation. Species are listed top to bottom according to phylogeny.

resulted in at least one successful trial, where pollen tubes were observed growing out of the cut style end. Surprisingly, the majority of heterospecific crosses also resulted in at least one successful trial. These trials represent pollen tube growth through heterospecific stigmas from species that diverged as much as 100 Mya (timetree.org; Kumar et al., 2022). Overall, pollen germination on the receptive stigma was not an absolute reproductive barrier for any species pairs in our study.

The best-fit model for our semi-in vivo pollen tube assays included pollen, but not pistil, species identity as a random effect. In other words, some species' pollen germinated more often on heterospecific stigmas than others, but species did not exhibit significant differences in the receptivity of their stigmas to foreign pollen. The slope of species-level pollen identity effects varied significantly (Figure 2C, D). Some species, such as *P. bracteosa*, *P. procera*, and *C. miniata* exhibit negative slopes, indicating that their pollen tends to perform better on close relatives and worse on distant relatives. While other species, such as *P. groenlandica* and *Lupinus* have positive slopes, indicating that their pollen performs better on distant than close relatives. The remaining species did not exhibit significant pollen-identity effects.

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In vivo pollen tube growth experiment

The phylogenetic distance between pollen and pistil also was not a strong predictor of the distance that pollen tubes grow through the style. Similar to the previous experiment, all heterospecific crosses (now including six species instead of 11) yielded at least some successful trials, demonstrating that pollen–pistil conflicts within the style do not form a complete reproductive barrier between any species pairs analyzed during pollen tube progression through the pistil. Pollen tubes from the shortest-styled species examined (*Lupinus*) were capable of growing to the end of the longest-styled species (*P. procera*). The majority of trials resulted in pollen tubes growing to the base of the style (i.e., to stage 4; Figure 3B). There was some variation in whether pollen was observed germinating and growing into the stigma (stages 1–2 of this assay), but if it did so, pollen tubes tended to grow the entire length of the style, that is, reach stage 4 as opposed to being arrested in stage 3 (Figure 3B). The best-fitting model showed no significant relationship between genetic distance and progression along the pollen tube pathway or significant species-level effects according to pistil or pollen identity (Table 1, Figure 3C).

Ovary response experiment

Ovaries changed in size in response to both con- and heterospecific pollen treatments (Figure 4). Measures of central tendency (i.e., mean, median), did not show consistent differences between pollinated and unpollinated treatments. Pollen treatments instead tended to result in some particularly large and some particularly small ovaries. Across all species, variation in ovary size usually increased in response to receiving a pollen treatment as compared to the unpollinated control (Figure 4). The pollen type that resulted in the highest variance relative to the unpollinated control was *Castilleja*, which also had consistently high rates of germination and growth through the pistil. The only exception to the trend of increase in variance of ovary area following pollination were the ovaries of *P. bracteosa*, which showed almost no change in response to either con- or heterospecific pollen (Appendix S1: Figure S2). Due to high heat and low precipitation late into the growing season relative to average, the *P. bracteosa* population used in this study experienced poor conditions during late summer of 2023. We hypothesize that the lack of response by *P. bracteosa* was due to environmental conditions, rather than true biological response to treatments. *P. bracteosa* pollen was viable though and produced pollen tubes and variance in ovary area was higher when applied to other pistil-types used here. Treatments on *P. bracteosa* ovaries were therefore excluded from further analysis.

We modeled ovary area as a function of genetic distance and conspecific cross areas using generalized linear models. We normalized area measurements according to unpollinated ovary area within each species so comparisons could be made across species. The best-fitting model showed a negative, but nonsignificant relationship between ovary size and relative phylogenetic distance between parent species (Table 1, slope = –0.758, $P = 0.129$) and significant pistil-identity random effects. There was a nonsignificant positive correlation between variance in ovary area following pollination and the average distance down the style that

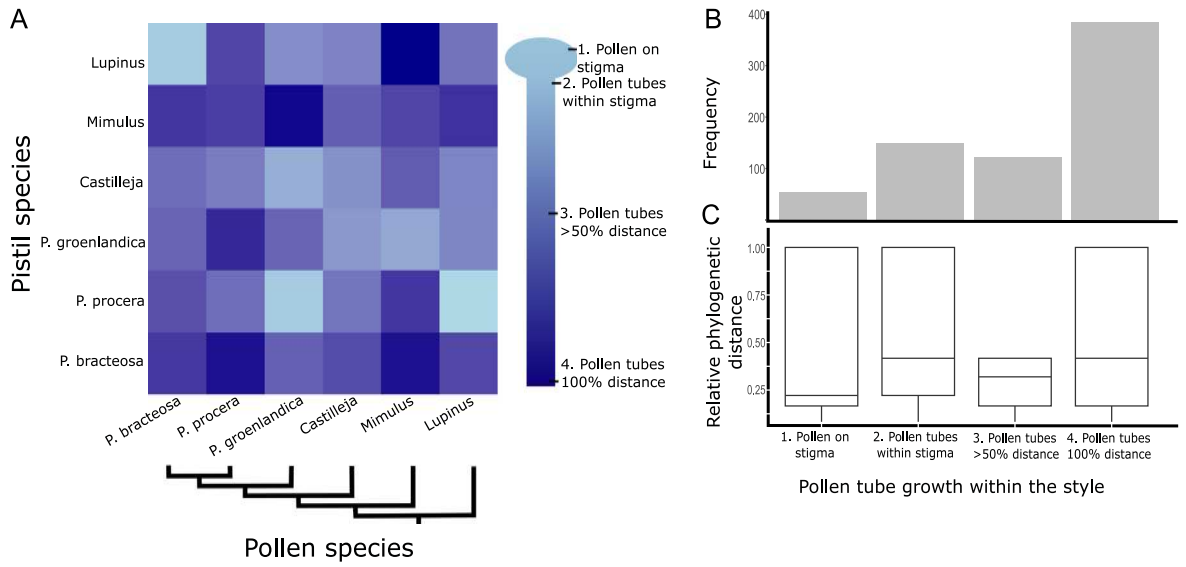


FIGURE 3 Results from in vivo pollen tube growth experiment. (A) The heatmap shows the average zone pollen tubes reach in reciprocal crosses, with phylogenetic relatedness shown in reduced phylogeny on the bottom. The colors of the squares were determined by averaging the scores (1–4) for pollen tubes within that cross, where (1) pollen was seen on the stigma, (2) pollen tubes germinated in the stigma, (3) pollen tubes grew halfway down the style, and (4) pollen tubes grew all the way down the style. (B) Histogram of the frequency of pollen tubes in each zone. In general, if pollen tubes emerged from the pollen grain (2), they tended to grow all the way down the pistil (4). (C) Boxplot showing the relationship between phylogenetic distance and the frequency of pollen tubes in each zone. The relationship was nonsignificant (slope = –0.091, $P = 0.78$).

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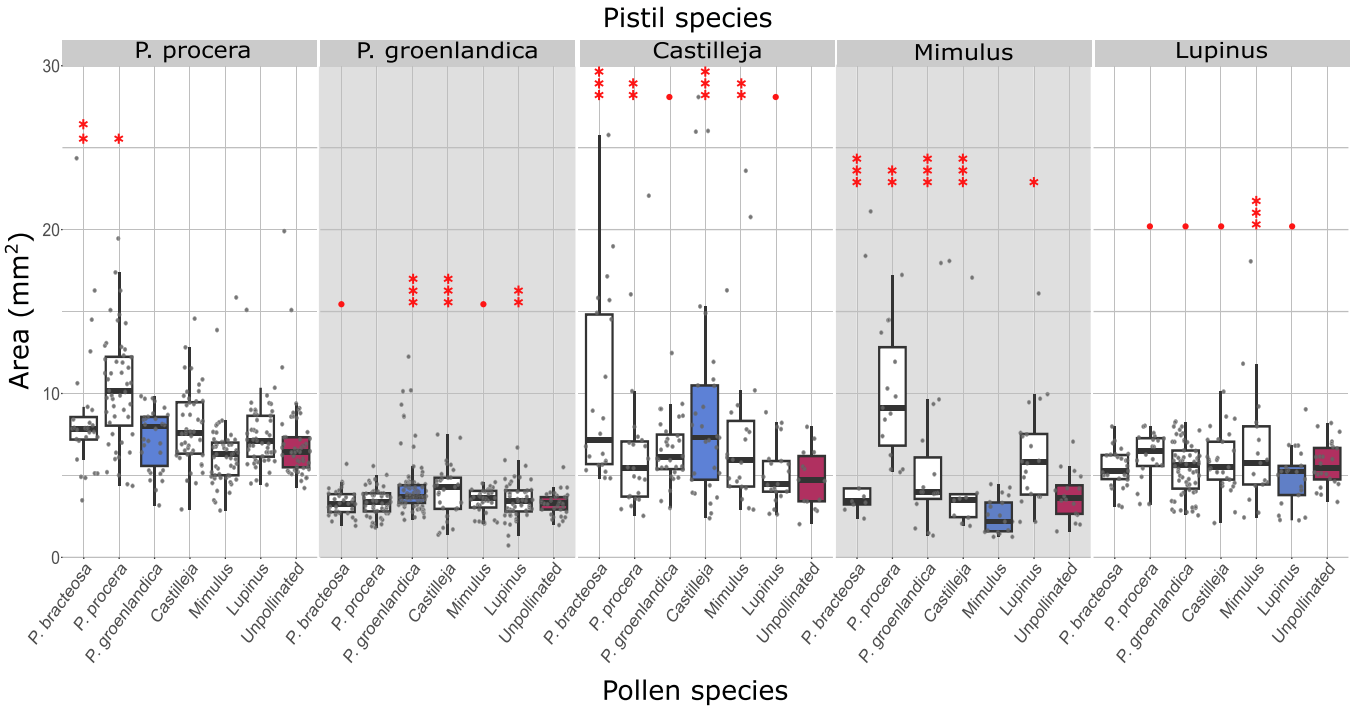


FIGURE 4 Results from the ovary measurement experiment. Boxplots show non-normalized ovary area measurements following pollen treatments (*x*-axis) within pistil types (subplots). Pistil and pollen species are ordered according to phylogeny (see tree in Figure 3). The unpollinated "control" treatments are shown on the right of each pistil subplot and highlighted in red for comparison. Conspecific crosses are highlighted in blue. Variances were compared using Fisher's *F* test. Crosses where the variance in area was significantly higher than in the unpollinated treatment are indicated with red asterisks (*significant, **highly significant, ***extremely significant), and crosses with variances quantitatively, but nonsignificantly, higher than the unpollinated treatment are indicated with a red dot. Exact values are reported in Appendix S2: Table S4. *Pedicularis bracteosa* ovary data is excluded from the boxplot because the flowers became desiccated before we could sample them due to unusual environmental conditions. Outliers above 30 mm^2 excluded.

pollen tubes tended to grow in cross types ($P = 0.0996$). We reiterate that the ovaries used in the ovary measurements experiment and pistils used in the fluorescence microscopy experiment came from the same flowers; crosses in which pollen tubes tended to reach the base of the style also generally exhibited higher variation in ovary size.

DISCUSSION

Pollen tube germination and tube growth in the style

We hypothesized that a decay in pollen tube growth with increasing phylogenetic distance between parental species would occur because of genetic incongruities in the pollen–pistil interaction, leading to mismatches in signaling and recognition systems. Many angiosperms have evolved the ability to distinguish between self, same species, and foreign tissue at the molecular level in response to other coevolutionarily antagonistic interactions. For example, the interaction between S proteins and SRNases in self-incompatible plants (Luu et al., 2000) or through the use of R genes during interactions with pathogens (Spoel and Dong, 2012). Additionally, the pistil uses molecular signaling, including repulsion signaling of pollen tubes, to block multiple pollen tubes from siring a single ovule (Mizuta et al., 2024). Many have suggested that plants can arrest heterospecific pollen tube germination at the stigmatic surface and throughout the style (Bedinger et al., 2017; Moreira-Hernández and Muchhala, 2019; Broz and Bedinger, 2021). Our study provides no evidence for the existence of molecular mechanisms that recognize foreign pollen or pollen tubes. We instead show that pollen tubes can successfully grow through pistils across great phylogenetic distances (>100 My divergent).

In a recent study, Streher et al. (2020) used tropical *Sisyrinchium* and *Fuschia* to track the fate of heterospecific pollen in intrageneric and interfamily crosses. They concluded that phylogenetic distance impacts heterospecific pollen tube growth, but not pollen germination. They noted, in fact, that in certain stigmas, heterospecific pollen germinated more often than conspecific pollen. Our results are consistent with this observation. We, however, offer a contrasting conclusion about within-style pollen tube growth: Phylogenetic distance between parental species does not significantly impact pollen tube length within the style (Figure 3). Consistency in results across the semi-in vivo (young styles) and in vivo (mature styles) experiments indicates that our results in the semi-in vivo pollen tube growth experiment reflect a true lack of barriers to heterospecific pollen germination and tube growth on stigmas and in styles.

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Variation in pollen germination was best explained by pollen species identity, not pistil species identity. Our data suggest that pollen varies in terms of the "vigor" with which the grains germinate on heterospecific stigmas, which may be the major determinant of whether or not heterospecific pollen can successfully germinate on stigmas. We found no evidence that stigmas prevent heterospecific pollen germination. All of the species used in this study are self-compatible, which is in contrast to the majority of evidence for the suspension of pollen-tube growth in foreign stigmas, which comes from species with self-incompatibility mechanisms in the context of the SIxSC rule (Hirose et al., 1995; Goodwillie and Ness, 2013; Baek et al., 2015). Our results suggest that in the absence of self-incompatibility mechanisms, suspension of pollen tube growth at the stigmatic surface does not act as a barrier to interspecific reproduction in many self-compatible species (SCxSC crosses).

Post-pollination, pre-zygotic barriers to reproduction

During conspecific crosses, pollen tubes tend to grow farther and faster in vivo than in vitro (Brink, 1924; Krichevsky et al., 2007). We expected the style to play an outsized role relative to pollen during intrastylar growth. The style secretes water and sugars, which have been shown to increase conspecific pollen tube growth rates (Wheeler et al., 2001), and stylar extracellular matrices "pull" and "guide" pollen tubes toward the ovary (Krichevsky et al., 2007). In our study, individual pollen species varied in how well they germinated on heterospecific stigmas, but once they penetrated stigmas, it was a race to the ovules (Figure 2B).

Nonexistent or very weak barriers to reproduction in the stigma and style imply a lack of specificity in the pollen–pistil interaction. Sanders and Lord (1989) showed that the stylar transmitting tract can translocate latex beads (nonbiological tissue) at similar rates to conspecific pollen tubes. In the absence of heterospecific pollen recognition, heterospecific pollen tubes should act like the latex beads in this study, which is what we found, suggesting that among the self-compatible species we studied, the style does not recognize heterospecific pollen tubes as "foreign" within the transmitting tract. Variation in stigma characteristics (i.e., "wet" or "dry" stigmas) could have additionally conferred some level of specificity: We did not expect pollen from wet-stigma species to germinate on dry stigmas because pollen grains would not be properly hydrated. Among our study species, there were no differences in pollen germination or tube growth based on "wet" or "dry" stigma classification (Heslop-Harrison and Shivanna, 1977; Appendix S2: Table S3). Instead of style mechanisms that arrest heterospecific pollen tube growth, selection may instead favor pollen characteristics that increase conspecific pollen tube success, like increased conspecific pollen tube growth rates.

Because our goal was to evaluate whether or not heterospecific pollen tubes are capable of reaching the base of the style, we left sufficient time for pollen tubes to grow through the style and did not directly measure pollen tube growth rates. Variation in pollen tube growth rates, though, is common among angiosperms. For example, pollen tube growth rates tend to be faster in longer-styled species relative to short-styled species (Lee et al., 2008; Williams, 2012). In *Pedicularis*, variation in style length has been suggested to act as a filter for heterospecific pollen tubes from short-styled species (Eaton et al., 2012; Tong and Huang, 2016). We therefore evaluated the relationship between style length and stigma depth (suggested to play a role in pollen tube growth success in *Pedicularis*; Wang et al., 2016) and pollen tube length within the style. We found that differences in style length did not impact pollen tube length in the style, though the relationship did trend positive (Appendix S1: Figure S3). The weak positive correlation between style length and heterospecific pollen tube length may indicate that over shorter time frames, style length and pollen tube growth rates may be significant factors that determine whether or not pollen tubes will make it to the end of the style.

Ovary response to con- and heterospecific pollen

In our experiments, treating stigmas with any type of pollen (conspecific or heterospecific) increased variation in ovary size (Figure 4). Focusing on variation rather than the central tendency of ovary size is consistent with what we know about ovary/ovule response to pollen in both viable and inviable crosses. Following viable fertilization events, ovules are known to either swell (Rifkin et al., 2023) or abort and shrivel (Nishiyama et al., 1991). In all genera used in this study (except *Castilleja*, not known), pollen tubes reach ovules in less than 24 h (Williams and Reese, 2019; Appendix S2: Table S3). Additionally, all species included in this study have multiflower inflorescences, some with very large inflorescences (more than 50 flowers) and multiple inflorescences per individual. In viable crosses, plants with multiple flowers can partition resources to certain ovaries instead of others or even to certain ovules and not others within the ovary (Marshall and Ellstrand, 1988; Marshall and Folsom, 1991; Tonnabel et al., 2021). Seed inviability often arises from sexual conflict of parent genes in a hybrid triploid endosperm, leading seeds to either shrink or swell beyond normal size (e.g., Coughlnn et al. 2020). Therefore, consistent with assumptions made regarding ovule swelling in interspecific crosses by Rifkin et al. (2023), we assumed that any change in ovary size relative to unpollinated controls indicated a response to a fertilization event. Ovaries responded to both conspecific and heterospecific pollen treatments, though we do not expect that heterospecific crosses would have resulted in viable seeds.

Pistil identity was only a significant predictor of the ovary response to pollination. Some heterospecific crosses yielded more extreme values of ovary growth than conspecific crosses, although both con- and heterospecific

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crosses exhibited higher variance in ovary size relative to unpollinated treatments (Appendix S2: Table S4). In *Mimulus*, we observed that ovaries tended to grow larger in heterospecific relative to conspecific crosses (Figure 4), which is consistent with the findings of Coughlan et al. (2020), who showed that ovules swell beyond average viable seed size in *Mimulus* in inviable hybrid crosses. We tested ovary response at a single time point in early development and did not directly observe growth of pollen tubes into the micropyle or otherwise validate that ovaries contained hybrid embryos. Follow-up work is needed to understand the mechanistic driver of ovarian response to heterospecific pollen and its implications on reproductive interference and hybridization.

Physical blocking and ovule usurpation hypotheses

Our finding that heterospecific pollen tubes are capable of growing through styles and fertilizing ovules in the absence of conspecific pollen, even when the resulting hybrid embryos are likely inviable, is significant in terms of our understanding of the role pollen tubes play in reproductive interference. In particular, it suggests that both mechanisms for reduced seed set following HPT—physical blocking and ovule usurpation—are possible. To be certain about the species identity of the pollen tubes we were observing, we used pure loads of heterospecific pollen. In nature, though, stigmas frequently receive mixed pollen loads with varying ratios of heterospecific to conspecific pollen grains.

In the alpine ecosystem we studied, pollinators tend to be generalists and exhibit inconsistent floral constancy. At least 50% of bees in the subalpine meadows surrounding Gothic, CO, carry pollen loads from multiple species at a given time (Brosi and Briggs, 2013). Briggs et al. (2016) additionally found that in *Delphinium barbeyi* (which shares pollinators with the species used in this study), 85% of stigmas had at least some heterospecific pollen, albeit sometimes at low heterospecific to conspecific pollen ratios (median ratio of 3:11 pollen grains per stigma). Heterospecific pollen grains, in fact, are often present on stigmas, but at variable frequency relative to conspecific pollen (Ashman and Arceo-Gómez, 2013). Heterospecific pollen is therefore consistently deposited on stigmas, but may be unlikely to exist in high enough density to physically block conspecific pollen or tubes (Ashman and Arceo-Gómez, 2013; Briggs et al., 2016). In contrast, ovule usurpation is not density dependent and could impose significant fitness costs to flowers regardless of the deposition ratio of heterospecific to conspecific pollen. We therefore propose that ovule usurpation may be a more common and significant cause of fitness loss induced by HPT than physical blocking.

Because ovule usurpation is very costly to the pistil species, we hypothesize that pre-pollination barriers to hybridization are favored by selection when they arise. Strong pre-pollination barriers could result in the low median number of heterospecific pollen grains on stigmas observed by Briggs et al. (2016). From studies that have quantified components of reproductive isolation between species pairs, we know that pre-pollination barriers tend to be of greater effect than post-pollination (e.g., within the stigma or style) barriers. For example, Ramsey et al. (2003) estimated that between co-occurring *Mimulus cardinalis* and *M. lewisii* in the Sierra Nevada Mountains, 97.6% of gene flow is prevented by pre-pollination reproductive barriers affecting pollinator fidelity. If the combined effects of pre-pollination barriers and differences in pollen tube growth rates can together allow for conspecific pollen to routinely outcompete heterospecific pollen, then there may in fact be little selective pressure on post-pollination mechanisms that could suspend foreign pollen in the pistil, despite their potential to protect ovules from usurpation. This hypothesis offers an explanation for our observation that stigmas and styles do not arrest heterospecific pollen germination and tube growth.

Increased specialization of plant–pollinator interactions has long been identified as a key adaptation in flowering plants that reduces HPT and increases reproductive isolation (Johnson et al., 2000; Brosi, 2016). Yet, generalist pollinators remain common in nature; in fact, all of the plant species in our study share generalist bee pollinators. Although specialization may benefit plants by reducing the potential for HPT, it is less commonly beneficial for pollinators to specialize. This antagonistic selection may therefore result in outcomes that can reduce HPT through alternative mechanisms that do not promote increased pollinator specialization. For example, traits that increase variation in pollen placement on pollinators' bodies (as in *Pedicularis*) or that increase floral constancy over short periods of time (a pollinator visiting one species many times between switching to another). Although these types of adaptations would still allow some HPT to occur, the impact would be reduced, perhaps significantly.

CONCLUSIONS

We present the surprising conclusion from evaluating semi-in vivo and in vivo crosses that heterospecific pollen tubes can germinate on stigmas and grow through styles and fertilize ovules, even when the pollen species and pistil species are very distantly related. This result has important implications for predicting the evolutionary consequences of HPT, particularly in terms of how selection is imposed on reproductive variation. If plants are receiving heterospecific pollen in high quantities, HPT can decrease seed set by physically blocking the stigma and style or by ovule usurpation, the latter of which is especially costly when the resulting hybrids are inviable, as is the case for the species examined here. We therefore hypothesized that given the lack of barriers to heterospecific pollen germination and pollen tube growth, selection imposed by HPT in diverse plant communities favors the evolution of pre-pollination barriers that decrease the probability of HPT occurring.

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We have mostly evaluated our results from the perspective of female fitness, but HPT also decreases male fitness through pollen loss to infertile crosses. Pre-pollination barriers that increase the probability of pollen being transferred within instead of between species should therefore additionally increase male reproductive success.

Finally, we concluded that pollen identity rather than style identity accounts for variation in heterospecific pollen germination. This conclusion is contrary to evidence that suggests the style should play an outsized role in pollen tube growth, though most work that has shown the importance of the style focuses on conspecific pollen–pistil interactions. Our results suggest that when HPT does occur, if heterospecific and conspecific pollen tube growth rates are similar, ovule usurpation is likely. Our conclusions therefore indicate a need for further work investigating variation in heterospecific pollen tube growth, especially with respect to pollen tube growth rates and the fertilization by heterospecific pollen tubes, while explicitly incorporating phylogenetic information.

AUTHOR CONTRIBUTIONS

R.O.C. and D.E. equally contributed to the idea and design of the study. R.O.C., A.C., and J.U.J. collected all data that was used in this study. R.O.C., D.E., and J.H.W. contributed to the methodological design of the study. R.O.C., D.E., J.H.W., and A.C. contributed to writing the manuscript.

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CONFLICT OF INTEREST STATEMENT

Joseph H. Williams is an Associate Editor of the *American Journal of Botany* but took no part in the peer-review and decision-making processes for this paper.

DATA AVAILABILITY STATEMENT

Data are available via the Dryad Repository: https://doi.org/10.5061/dryad.qv9s4mwqr.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supplemental figures.

Figure S1. Boxplots showing distribution of ovary lengths and widths measurements.

Figure S2. Complete boxplot of ovary area measurements, including *P. bracteosa*.

Figure S3. Correlation between pollen tube growth and difference in style length between pollen donor and pollen recipient.

Appendix S2. Supplemental tables.

Table S1. Accessions and citations for the ITS sequences used to calculate relative phylogenetic distance.

Table S2. Minimum and maximum set on node ages during time calibration of the phylogeny.

Table S3. Information about stigma and pollen characteristics in study species.

Table S4. Comparison of ovary area variance in pollinated and unpollinated treatments.

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Appendix 1 Herbarium voucher information for all study species. Two specimens were collected per species. One was deposited in the Rocky Mountain Biological Laboratory (RMBL) herbarium, which we report here. If multiple voucher numbers are listed in the RMBL column, the specimen was too large and had to be split onto multiple vouchers. Every voucher associated with that individual is listed. We deposited the second individual that we collected of each species in the New York Botanical Garden William and Lynda Steere Herbarium. Due to a backlog of vouchers, our specimens had not yet been processed at the time of publication. They will be able to be found using the collector's and species names (R. Cohen) after they have been fully processed by NYBG.

Species	Personal collection no.	RMBL voucher
Agastache urticifolia	RC15	RMBL0007643
Castilleja miniata	RC03	RMBL0007647
Castilleja sulphurea	RC11	RMBL0007644
Linum lewisii	RC07	RMBL0007648
Lupinus argenteus	RC17	RMBL0007645
Mimulus guttatus	RC09	RMBL0007649
Pedicularis bracteosa	RC05	RMBL0007642
Pedicularis groenlandica	RC01	RMBL0007650
Pedicularis parryi	RC13	RMBL0007646
Pedicularis procera	RC19	RMBL0007638, RMBL0007639, RMBL0007638
Pedicularis racemosa	RC21	RMBL0007641

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